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# NONSEGMENTED NEGATIVE-STRAND RNA VIRUSES: Genetics and Manipulation of Viral Genomes

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## ABSTRACT

Protocols to recover negative-strand RNA viruses entirely from cDNA have been established in recent years, opening up this virus group to the detailed analysis of molecular genetics and virus biology. The unique gene-expression strategy of nonsegmented negative-strand RNA viruses, which involves replication of ribonucleoprotein complexes and sequential synthesis of free mRNAs, has also allowed the use of these viruses to express heterologous sequences. There are advantages in terms of easy manipulation of constructs, high capacity for foreign sequences, genetically stable expression, and the possibility of adjusting expression levels. Fascinating prospects for biomedical applications and transient gene therapy are offered by chimeric virus vectors carrying novel envelope protein genes and targeted to defined host cells.

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## INTRODUCTION

Negative-strand RNA viruses (NSV) are enveloped viruses of hosts from throughout the animal and vegetable kingdoms. They differ widely in morphology and host interactions, and have varied genome structures. There are astonishingly old species, e.g. rabies virus, which have been known for centuries, but there are also emerging viruses adapting rapidly to new hosts, such as phocine distemper virus, equine morbilli virus, or Ebola virus. Their genomes may be single RNA molecules (*Mononegavirales* order, nonsegmented negative-strand RNA viruses) (142, 143), or made up of multiple RNA molecules (viruses with a "segmented" genome) (124). Despite this diversity, it appears that they have originated from a common ancestor (143, 175). One particular feature common to all negative-strand RNA viruses is their mode of replication and transcription. Their genetic information is exclusively found in the form of a tight, helical ribonucleoprotein complex (RNP). Only the RNP, not the naked RNA, is a suitable template for replication of RNPs as well as transcription of translatable, free mRNAs (7, 67). Unlike cores, capsids, or nucleocapsids of positive-strand RNA viruses or DNA viruses, or eukaryotic chromatin structures, RNPs apparently never disassemble and RNA synthesis does not change the structure of the RNA-N protein template. The RNP proteins are actively required for any gene expression and can be regarded as part of the template, like the RNA itself, or as part of the polymerase. In essence, the genome of a NSV is not an RNA, but an RNP.

The original distinction between positive- and negative-strand RNA viruses is based on the fact that denatured RNAs of negative-strand viruses cannot initiate an infectious cycle after transfection of appropriate host cells (6). Neither the negative-strand (i.e. the RNA is complementary to mRNA strand) genome RNA found in extracellular virions nor the complementary "positive-" strand complement can be translated by ribosomes to form the critical virus polymerase. To be biologically active, the RNAs must at least be encapsidated into

a virus nucleoprotein (N) to form an RNP. The simplest fully active holo-RNPs are known from rhabdoviruses like vesicular stomatitis virus (VSV) or rabies virus, and contain only three virus-derived proteins, the nucleoprotein (N), the RNP-dependent polymerase (L), and a phosphoprotein (P) acting as polymerase cofactor. Indeed, VSV RNPs are transcriptionally active in the test tube after addition of ribonucleotides and appropriate ions. In total, VSV and rabies virus genomes encode only five proteins. In addition to the three RNP proteins, there are two envelope-associated proteins involved in getting the RNP into and out of host cells, an internal matrix protein (M), and a transmembrane glycoprotein (G) (178). When enveloped viruses are regarded as highly evolved liposomes, providing a membrane targetable to cell surface receptors and a payload compartment containing the viral genetic "information," such rhabdoviruses are very close to a putative "minimal" enveloped virus. Because of these features, VSV has for decades provided an extraordinary model for several aspects of virology, such as transcription and virus assembly. Other RNP viruses have been useful as models for other aspects. For example, detailed analyses of influenza virus cell entry have brought forth sophisticated knowledge on activation of fusogenic proteins and membrane fusion.

Unfortunately, the lack of systems for genetic manipulation of RNP viruses using powerful recombinant DNA technologies has long limited experimental approaches to studying the genetics and biology of negative-strand viruses. This has also prevented the enormous potential of RNP viruses from being exploited as tools for basic and applied biomedical research. This potential stems from the high integrity of RNP genomes within the cell, the mode of gene expression from simply organized genomes, the cytoplasmic replication cycle of most of RNP viruses, and also from the relatively simple structure of envelopes and holo-virions.

Techniques to introduce recombinant RNA into the RNP genome of a negative-strand RNA virus were first described for the segmented influenza virus (61, 110). Several years later, in 1994, the first nonsegmented RNP virus, rabies virus, succumbed to genetic engineering (157), followed shortly thereafter by VSV and members from all paramyxovirus genera (Table 1). Recently, the first member of the segmented Bunyavirus family was also recovered from cDNA (21), so that members of most major RNP virus groups are now amenable to genetic manipulation. By using recombinant DNA technology, defined virus mutants can now be designed to elucidate the basic common principles of RNP gene expression, as well as individual sophisticated features, such as the utilization of overlapping reading frames, RNA editing, RNA splicing, or ambisense gene expression. In addition, viruses are being generated that have never existed in nature; that infect novel target cells, or express foreign proteins. Thus far, RNP virus research has mostly been aimed at preventing infections

Table 1 Recovery of negative-strand RNA viruses from cDNA

		Reference
Rhabdoviridae		
Rabies virus	<i>Lyssavirus</i>	157
VSV	<i>Vesiculovirus</i>	105
VSV	<i>Vesiculovirus</i>	182
Paramyxoviridae		
Measles virus	<i>Morbillivirus</i>	146
Sendai virus	<i>Paramyxovirus</i>	69
hRSV	<i>Pneumovirus</i>	34
Sendai virus	<i>Paramyxovirus</i>	91
Rinderpest virus	<i>Morbillivirus</i>	11
hPIV-3	<i>Paramyxovirus</i>	81
hPIV-3	<i>Paramyxovirus</i>	57
SVS	<i>Rubulavirus</i>	78
Nonsegmented virus		
Bunyaamwera virus	<i>Bunyaviridae</i>	21

with those agents. It is a reflection of the development of RNP virology over the past decade that we may now start thinking of ancient foes as friends.

This article concentrates on nonsegmented NSV (NNSV). This group is now vigorously entering the new world of recombinant DNA, and there are many examples to show that the predicted potential is real. This chapter does not include an exhaustive description of particular properties and molecular mechanisms within each family, group, or subgroup, since excellent reviews on paramyxoviruses (67, 104), rhabdoviruses (139, 178), filoviruses (63, 167), and bornaviruses (43, 171), and on reverse genetics of the segmented influenza virus (69, 128) are readily available elsewhere. The focus is rather on recent developments that contribute to our understanding of the principles of negative-strand RNA virus genetics and illustrate the possibilities and prospects offered.

## CLASSIFICATION

The known viruses with monopartite negative-sense RNA genomes constitute the order Mononegavirales and are classified in four families, the *Rhabdoviridae*, *Paramyxoviridae*, *Filoviridae*, and *Bornaviridae* (124, 142, 143).

The latter two families are represented by a single genus. The genus *Bornavirus* contains a single member (species) infecting vertebrates. Its genome organization is reminiscent of that of rhabdoviruses, but unlike rhabdoviruses of vertebrates, it replicates in the nucleus of infected cells. Its gene expression involves features otherwise known only from segmented viruses, such as mRNA

splicing (43, 171). Four species, including Ebola virus and Marburg virus, have been defined in the genus *Filovirus*, based on nucleotide sequence and antigenic divergence and a differential manner of expressing the attachment (G) protein.

The *Rhabdoviridae* comprise five genera, differentiated on the basis of host range, presence of supplementary genes, and the intracellular site of virus replication. Rhabdoviruses are perhaps more widely distributed in nature than any other virus family (178). They infect vertebrates and invertebrates, as well as many species of plants. Except for rabies virus and some fish rhabdoviruses, which appear to be confined to vertebrates, all other rhabdoviruses, including VSV, are thought to be transmitted in nature by infected arthropods, which may be the original hosts from which all rhabdoviruses evolved. Rhabdovirions have a typical bullet-shaped morphology (approximately 170 × 80 nm), except for certain plant rhabdoviruses, which are bacilliform in shape. Rhabdoviruses replicate in the cytoplasm of infected cells and virions mature by budding predominantly from the cell surface. However, some plant viruses have a nuclear life cycle and bud from the inner nuclear membrane. Rhabdoviruses that infect humans have been classified into two genera: the *Vesiculovirus* genus, stemming from vesicular stomatitis virus (VSV), and the *Lyssavirus* genus, comprising rabies and rabies-related viruses.

The family *Paramyxoviridae* (67, 104) was reclassified in 1993 into two subfamilies: the *Paramyxovirinae*, with three genera of viruses, and the *Pneumovirinae*, represented by two genera (124). Paramyxoviruses are generally spherical and 150 to 400 nm in diameter, but can be pleiomorphic in shape. Morphological distinguishing features are size and shape of RNPs, whereas biological criteria are antigenic cross-reactivity between members of a genus, and the presence or absence of neuraminidase activity. Pneumoviruses are further distinguished by the number of encoded proteins and an attachment protein (G) that is very different from that of *Paramyxovirinae*.

Paramyxoviruses are found in vertebrate hosts; most use sialic acid-containing cellular receptors for entry into target cells and they cause respiratory disease (Pneumo-Paramyxo-, Rubulavirus). Replication of *Paramyxoviridae* takes place exclusively in the cytoplasm.

The degree of nucleotide sequence homology, especially in the domain III region of the polymerase molecule (141), is consistent with a phylogenetic relationship among at least three of the four families making up the order Mononegavirales (9) and correlates well with the conventional classification.

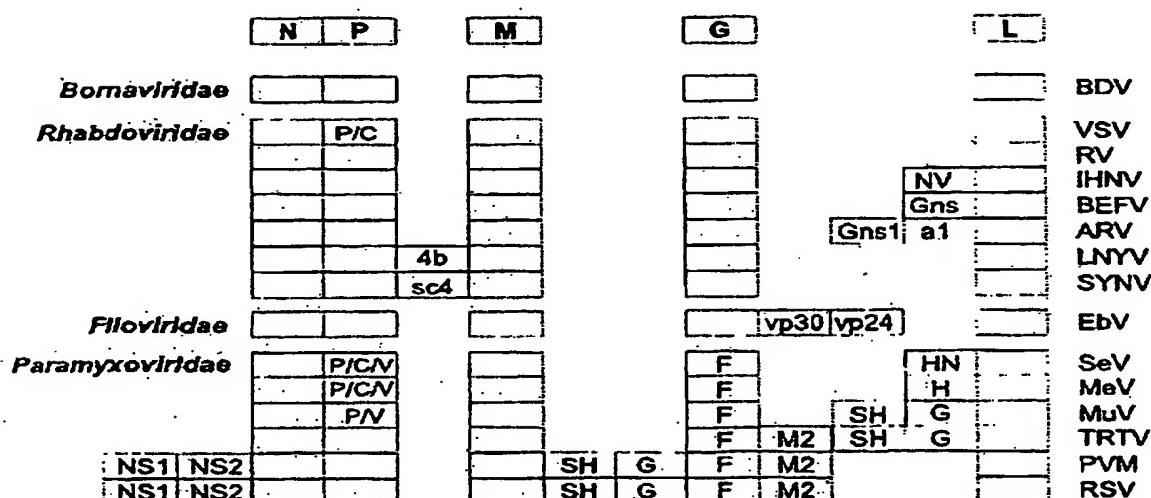
## THE PATTERN OF GENOME ORGANIZATION

The structure and the organization of the genomes of nonsegmented NSV (NNSV) is identical, and is governed by the particular mode of gene expression. The linear, single-strand RNAs of 11–19-kb size represent a succession

of individual protein-encoding genes, each defined by an upstream gene start signal and downstream gene end signal. The 3' and 5' ends of the RNA are represented by short nontranslated sequences that carry important *cis*-acting signals for transcription and replication. Transcription starts at the very 3' end of the genomes producing an approximately 50-nucleotide leader RNA. This is followed by sequential synthesis of the individual mRNAs, giving rise to a gradient of transcripts, steadily decreasing toward the template 5' end. Thus, the gene order roughly reflects the required amount of products.

The five basic genes of nonsegmented RNP viruses (N, P, M, G or analogue, and L) (Figure 1) are maintained in highly similar order (143, 175). As a

#### Gene order of nonsegmented negative-strand RNA viruses



**Figure 1** Gene order of nonsegmented negative-strand RNA viruses. Comparison of representative negative-strand RNA virus genomes. Equivalents of the five basic genes are drawn as filled boxes: N (NP in paramyxoviruses; nucleoprotein), P (phosphoprotein), M (matrixprotein), and L (Large; catalytical subunit of the polymerase). The P gene-encoded C- and V-proteins of paramyxoviruses are expressed from alternative initiation codons or by RNA editing, respectively (see text). The fusion protein F of paramyxoviruses is aligned with G protein from rhabdoviruses that has fusion and attachment activity; both are type I membrane proteins. The G proteins of paramyxoviruses represent type II membrane proteins. Rhabdoviruses possess duplicated, nonfunctional G genes (Gns, Gns1, a1) downstream of G and proteins of unknown function (4b, sc4, NV) between P and M or downstream of G. For details see text. Abbreviations: BDV, bovine disease virus; VSV, vesicular stomatitis virus; RV, rabies virus; IHN, infectious hematopoietic necrosis virus; BEFV, bovine ephemeral fever virus; ARV, Adelaide river virus; LNYV, lettuce necrotic yellows virus; SYNV, sonchus yellow net virus; EbV, Ebola virus; SeV, Sendai virus (murine parainfluenza virus type 1); MuV, mumps virus; MeV, measles virus; TRTV, turkey rhinotracheitis virus (avian pneumovirus); PVM, pneumovirus of mice; RSV, respiratory syncytial virus.

corollary, the noncatalytical RNP proteins, which are needed in stoichiometric amounts, the nucleoprotein (N; NP in paramyxoviruses), and the phosphoprotein (P; formerly also NS) are encoded in the 3' proximal part of the genome, whereas the catalytical subunit of the polymerase (L) is located 5' terminally. N protein mRNAs are therefore the most abundant mRNA species, whereas transcripts encoding L, which is required in catalytic amounts, are much less. In between these two invariable blocks are located genes coding for matrix (M, VP40) and transmembrane envelope proteins (G, GP, F, HN). The fusion proteins (F) of paramyxoviruses are structurally similar to G and GP proteins in representing type I transmembrane glycoproteins, suggesting a relationship. The attachment proteins of paramyxoviruses (H, HN, G), which are mostly encoded downstream of F, are type II transmembrane glycoproteins and often contain a neuraminidase function (HN, hemagglutinin-neuraminidase). Insertion of additional genes in NNSV genomes is mostly observed within the envelope gene block, or upstream of the L gene. These include genes encoding proteins of unknown function, such as sc4 of sonchus yellow net virus (79, 158), NV (nonviral) in some fish rhabdoviruses (101), or nonfunctional G duplicates, as for BEFV (179).

The most atypical gene organization, both with regard to the order of genes and the presence of genes with no homologues in other members of the order, is found in members of the Pneumovirinae subfamily of the Paramyxoviridae (143). As compared to Paramyxovirinae, the order of genes is maintained in the avian pneumovirus (APV), but a novel nonstructural gene (M2) and an additional envelope protein gene (SH) are located between the M and F genes. A particular feature is displayed by RSV and pneumonia virus of mice (PVM), which are both closely related to APV, as established by nucleotide sequence comparison. This is the presence of two genes upstream of the N gene. These genes encode two nonstructural proteins (NS1, NS2), which are expressed abundantly, according to their 3' proximal location. Moreover, the envelope protein genes SH and G of RSV and PVM have changed location with F and M2, with respect to the gene order of APV. The M2 gene of pneumoviruses is unique in containing two overlapping open reading frames. The product of one of the ORFs (M2-1) encodes a protein involved in transcription elongation (35).

Generally, the genes of NNSV represent single cistrons containing a single open reading frame that is transcribed colinearly and gives rise to one protein. However, in a number of viruses, there is one gene featuring a quite opposite strategy. Sophisticated mechanisms allow the expression of up to five individual proteins from the P gene of members of the Paramyxovirinae subfamily. In addition to open reading frames internal to the P open reading frame (C, X), a unique RNA-editing mechanism involving the addition of nontemplated G

residues provides proteins colinear to P in the aminoterminal moiety while having different carboxyterminal parts (V, W, I). Many of these proteins may have accessory functions in certain stages of transcription and replication, which can now be addressed with the help of recombinant virus mutants (see below).

## VIRUS STRUCTURE AND VIRUS PROTEINS

Despite their wide range of morphological types, all NNSV are composed of two major structural components: a helical ribonucleoprotein core (RNP) and an envelope in the form of a lipoprotein bilayer membrane, more or less closely surrounding the RNP core.

### *RNP Proteins*

In the RNP, the genome RNA is always tightly encased by nucleoprotein (N) to form a helical, left-handed coil. Virtually no sequence homology is found between the N proteins of different genera, but the overall structure of the N protein seems to be similar. N does not appear to be a classical RNA-binding protein, in that it does not contain any previously described RNA-binding motifs, nor does it interact with RNA in Northwestern blots.

Both rhabdo- and paramyxovirus RNPs are remarkably stable and tight, as they withstand high salt and gravity forces in CsCl centrifugation, banding at 1.29–1.31 g/ml, and protect the RNA against access by small molecules such as RNases (16, 80). This indicates that strong hydrophobic forces maintain the N-RNA and N-N interactions. For the tightly encapsidated RNA to serve as a template for the polymerase, structural transitions would be required that bring the RNA to the surface. Alternatively, local displacement of N subunits might occur, somehow similar to the separation of two DNA strands during transcription (126).

As first predicted by calculating images of Sendai virus RNPs, each N protein is associated with exactly six nucleotides (58). The analysis of artificial and natural virus genome sequence lengths, and the observation that genomes replicate much more efficiently when they comprise an exact multimer of six in length, established the importance for replication of the so-called "rule of six" (27). Calain & Roux proposed that the 3' ends of the RNP RNAs are efficient templates for replication only when they are precisely covered with an N subunit. This implies that the viral polymerase (P and L) is interacting with the template bases for RNA synthesis in a defined context with the N subunit and that the N protein itself might be regarded as part of the template. The rule of six applies to members from all genera of the paramyxovirinae subfamily (57, 126, 135, 146, 149). In contrast, there appears to be no rule of (n) for the related pneumoviruses (148), or the rhabdoviruses, although the N proteins of the latter have been predicted to be associated with 9 nucleotides (174).

Paramyxovirus N proteins range from 489–553 amino acids. The N-terminal 80% of the protein is relatively well conserved between related viruses, whereas the C-terminal 20% represent a hypervariable domain, which is likely to extend from the globular body of the protein, and which contains most phosphorylation sites. For assembly of paramyxovirus RNPs, which contain approximately 2600 N molecules, the C-terminal hypervariable domain is dispensable (22, 46, 47, 127). However, RNPs made up of such truncated proteins may not serve as templates for the polymerase, suggesting that the C terminus of N is involved in binding P or complexes of P and L protein (46, 48). Rhabdovirus N proteins consist of approximately 420 amino acids; approximately 1300 N molecules are required for encapsidation of the 12-kb RNA of VSV.

Two additional proteins are associated with the RNP structure, the phosphoprotein P (300–500 molecules per virion), named for its highly phosphorylated nature, and the large (L) protein (~50 molecules per virion), a multifunctional protein containing the catalytical polymerase activities.

P proteins of NNSV are variable in length (245–603 aa). P is a modular protein that plays a crucial role in all RNA synthesis. It is present both as a homotrimer (45, 68) and in complexes with other proteins. Together with L it forms the active polymerase for both transcription and replication (8, 60, 74), and together with unassembled N it forms a complex involved in encapsidation of RNA during genome replication (82). It has also been suggested that this complex prevents N from assembling RNA nonspecifically (47, 112).

The L (large) protein is the least abundant of the structural proteins. The L genes of many NNSV have now been sequenced and they are all of very similar length (~2,200 aa). There are five short regions of high homology near the center of these proteins, which are conserved in RNA-dependent RNA polymerases of any virus (141) and include the catalytical polymerase site (155). In addition to RNA synthesis (elongation, polyadenylation, see below), the P-L protein complex of NNSV is enzymatically active as a guanylyl- and methyl-transferase during capping of mRNAs (10). A kinase activity has also been attributed to the L protein, but in the absence of L, P proteins of NNSV are phosphorylated by cellular kinases (52, 75), and the relevance of the L kinase for virus replication is uncertain.

### *Envelope Proteins*

The matrix protein is the most abundant protein in the virion. M proteins contain 341–375 residues. They are basic proteins and are somewhat hydrophobic, although there are no domains of sufficient length to span a lipid bilayer, and they are translated on free cytoplasmic ribosomes. Many M proteins interact directly with membranes and are found associated with RNPs (30, 165). The proteins probably contain amphipathic alpha-helices that insert themselves into the inner leaflet of a lipid bilayer and make up the contact with the helical RNP.

Evidence has been obtained that the M proteins of paramyxoviruses and rhabdoviruses interact specifically with membranes in which the respective surface glycoproteins of those viruses are integrated (161; R Cattaneo, submitted; T Mebatson, submitted).

There is clear evidence that the association of rhabdovirus M protein with RNP shuts down virus transcription (33). Most likely this is associated with preparing the structure of RNPs for budding. In the presence of M, relaxed and transcriptionally active RNPs are condensed to form the typical cigar-like rods found in mature bullet-shaped virions. Moreover, RNPs surrounded by M protein are able to efficiently bud spikeless, noninfectious bullet-shaped virions off the cell surface (116). These features make at least the rhabdovirus M the central protein of virus assembly, morphogenesis, and budding, i.e. it is the protein "pulling it all together and taking it on the road" (134).

The M protein of paramyxoviruses may have a largely corresponding role. Paramyxovirus particles have a more flexible shape than rhabdovirions, and the RNPs within the virions appear more loosely associated with the M layer of the envelope. Also, more than one RNP may be enclosed into a virus particle (92), whereas polyploidy is not observed in the inflexibly shaped rhabdoviruses. It is presumed that shut-down of paramyxovirus transcription by M is also associated with preparing RNPs for export. An important role in virus budding is suggested by the observation that M proteins are often inactivated in persistent virus infections where budding fails to occur. For example, in subacute sclerosing panencephalitis (SSPE), a fatal disease induced by measles virus, the M protein is absent for a variety of reasons, or is not associated with budding structures and is not able to bind to RNP structures (14). In addition, the M protein appears to impose a regulated structure of transmembrane glycoproteins, probably by providing a lattice for their cytoplasmic tails. Interestingly, the absence of M seems to result in an increased, uncontrolled membrane fusion activity (R Cattaneo, submitted).

The surface of NNSV virions contains spikelike projections, which are responsible for getting the virus into an appropriate host cell. Rhabdoviruses have a single glycoprotein (G) that forms ~400 trimeric spikes tightly arranged on the virus surface. The protein is a typical type I transmembrane protein with the majority of residues exposed on the surface. A hydrophobic domain spans the membrane, and a short cytoplasmic domain extends into the virion. The protein is synthesized as a precursor from which a short N-terminal signal sequence is cleaved after the protein is inserted into the endoplasmic reticulum. The VSV G protein has served as an important model for protein folding assisted by ER-resident chaperones, glycosylation, assembly, and transport to the cell surface (3, 50, 113), where RNPs, M, and G meet to venture virus assembly and budding of cell-free virus particles.

Upon binding to a cellular receptor (the identity of which is not known for any rhabdovirus), uptake of bound virions by endocytosis, and the associated drop in pH (below 6.1), conformational changes occur in the G trimers. This change presumably exposes an internal hydrophobic loop that can insert into a target membrane and mediate membrane fusion.

Like the rhabdoviruses, Filoviridae and Bornaviridae possess a type I transmembrane protein (GP), mediating both attachment and membrane fusion at acidic pH. In contrast to rhabdovirus Gs, the fusion property of these proteins is activated by proteolytic cleavage, giving rise to a linear hydrophobic fusion domain that is typical of fusion proteins of most viruses, including the most thoroughly studied HA of Influenza A virus, the envelope protein of HIV, as well as the F proteins of paramyxoviruses (103).

All *Paramyxoviridae* possess at least two integral membrane proteins, one involved in cell attachment (H, HN, G) and the other in pH-independent membrane fusion (F). Similar to the proteins described above, F is a type I transmembrane protein, which is activated by proteolytic cleavage, whereas the attachment proteins are "novel" type II membrane proteins, which have the N terminus extended into the virus particle. An analogy of the F with rhabdovirus G is further emphasized by the observation that there are trimeric F protein assemblies, while the attachment protein spikes appear to be organized in tetramers.

The attachment proteins of parainfluenza viruses and rubulaviruses bind to cellular sialic acid-containing proteins or glycolipids. The affinity of binding is high enough to cause agglutination of erythrocytes (hemagglutination) by these viruses. The proteins also have neuraminidase activity and have been designated hemagglutinin-neuraminidase (HN). The Morbillivirus attachment protein has some hemagglutination activity, but lacks neuraminidase activity and is called (H), although at least in some cases sialic acid is not the primary receptor structure for those viruses. For measles virus, CD46 has been identified as one of apparently several alternative receptor proteins.

After correct attachment of a particle to a host-cell receptor, the paramyxovirus envelope lipid bilayer fuses directly with the host cell plasma membrane by virtue of the action of F (103). A prominent feature of paramyxoviruses is syncytium formation in cell culture. Interestingly, the presence of an attachment protein does not seem to be absolutely required for cell entry in some paramyxoviruses (88). Also the SH membrane protein of mumps virus and of RSV is not required for virus propagation (24, 88, 168).

### *Accessory Proteins*

**C PROTEINS** The P gene of paramyxovirinae encodes a nested set of small accessory proteins (C', C, Y1, Y2), referred to collectively as C proteins. They are encoded in the P+1 frame and are generated by initiation at alternative

translation initiation codons. C proteins are characterized by a positive charge, suggesting possible interaction with RNA. In vitro studies indicate that the C protein of Sendai virus might be involved in promoter-selective down-regulation of RNA synthesis (25, 171). As established by the recovery of recombinant C-deficient Sendai and measles viruses, C proteins do not represent essential gene products. While a measles C-negative mutant multiplied in tissue culture without obvious impairment (145), replication of the C-deficient Sendai virus was severely affected. Moreover, the virus was unable to multiply in vivo and to cause pathogenesis (102).

Basic C and C'-like proteins are also encoded by the rhabdovirus VSV (136, 162), but not by members of the lyssavirus genus including rabies virus. The addition of purified C' protein to in vitro transcription had suggested a stimulation of quantity and quality of mRNAs (136), while growth of a recombinant C-deficient VSV in cells culture was not distinguishable from wild-type virus (97).

**V PROTEINS** Of the proteins generated by RNA editing (see section on transcription, below) from the P gene of paramyxoviridae (V, W, I), the V protein is almost universally conserved in all three genera. V proteins are characterized by a cysteine-rich domain at their C-terminal halves, which are fused to the N-terminal halves of P proteins. The V protein of Sendai virus inhibits DI genome replication, possibly interfering with the RNA encapsidation step, but appears not to affect mRNA synthesis (44, 83). Recombinant V(–) Sendai viruses propagated in cell culture as efficiently or better than wt virus, with regard to gene expression, replication, and cytopathogenicity (53; 54, 89). The V(–) viruses, however, showed markedly attenuated pathogenicity for outbred mice (54, 89). The pathogenicity determinant was mapped to the cysteine-rich domain of the protein (90). A recombinant measles virus deficient for editing and thus unable to produce V protein was also found to be viable in cell culture (149).

**NS-PROTEINS OF PNEUMOVIRUSES** The function of NS1 and NS2 proteins is not well established. At least the NS2 protein of RSV is a nonessential gene, as an autonomously replicating NS2-deficient virus could be recovered from cDNA (P Collius, submitted). After NS1 was expressed in a model genome system, an efficient inhibition of RNA synthesis was observed (2).

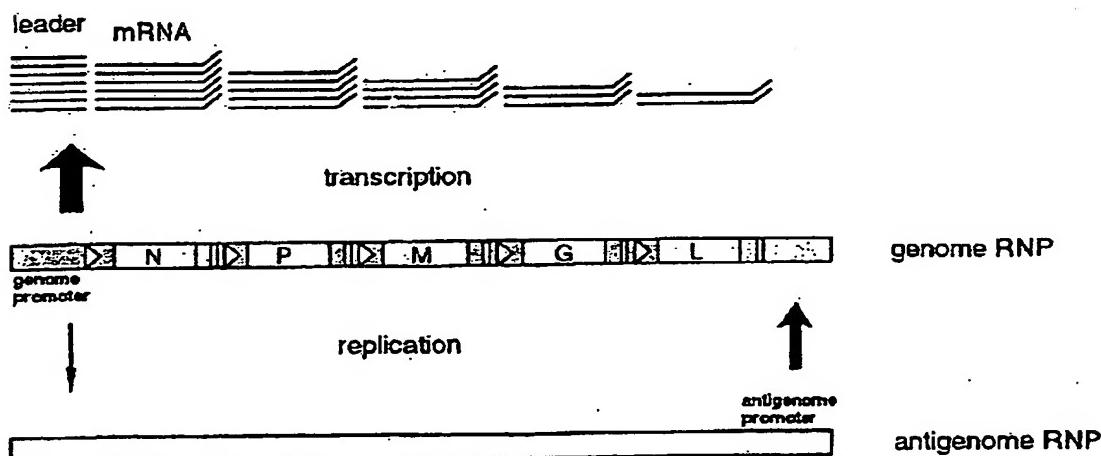
## GENE EXPRESSION OF NEGATIVE-STRAND RNA VIRUSES

### *The Stop/Start Mechanism of Transcription*

It is now nearly 30 years since Baltimore et al (6) discovered that VSV packages within the mature virion the viral RNA polymerase that transcribes the genome

into mRNAs. This observation enabled VSV gene expression to be studied in cell-free extracts (60) and made VSV an early paradigm for NNSV gene expression [reviewed in (7)]. It was shown that both P and L proteins were required for mRNA synthesis from the N-encapsidated RNA, a finding that applies to all NNSV analyzed so far. Two important features are sequential transcription and polarity (1). Kinetic studies demonstrated that synthesis of the transcripts follows the order of the genes in the genome: 3'-Ie-N-P-M-G-L-5' (5, 7, 86). Thus, the leader RNA and the N mRNA are the first and the L mRNA is the last to be transcribed. In addition to this temporal order, there is a gradient in the quantity of transcripts, following the same 3' to 5' order. These observations have led to the so-called stop-start model of transcription, according to which the polymerase enters the RNP-RNA exclusively at the 3' end and transcribes the genes successively in a 5' direction (59). Data from other nonsegmented RNP viruses confirmed the polar transcription as a common principle (Figure 2). So far, the stop-start mechanism best accommodates all data, although internal initiation at gene borders has not yet been definitely excluded (31, 32, 159).

The *cis*-acting transcription signals defining a gene border have been identified for many NNSV by comparing the sequences present in the genome



*Figure 2* RNA synthesis of nonsegmented negative-strand RNA viruses. The genome organization of a simple rhabdovirus containing the five minimal genes N, P, M, G, and L (*open boxes*) is shown. Transcriptional start and stop/polyadenylation signals are indicated by *open arrowheads* and *vertical boxes*, respectively. Starting from the genome promoter at the 3' end of the viral RNA (RNP), transcription yields successively a noncapped and nonpolyadenylated leader RNA, and five capped, polyadenylated mRNAs. Attenuation at gene borders produces a gradient of transcripts. Replication requires polymerase proteins and N protein and yields a full-length antigenome RNP, which directs replicative synthesis of genome RNPs. Arrows indicate relative activities of promoters (for details see text).

template and in the mRNA ends. The mRNAs of a virus start with common short consensus sequences that are templated by the genome, and which were regarded as gene start signals. The first A residues of the poly(A) tails of mRNAs match a short stretch of U residues in the genome that is regarded as the transcription stop/polyadenylation signal. At the junction between two genes, the stop/polyadenylation signal and the start signal are usually separated by several nucleotides (1–59 residues) that are not present in the mRNAs, the so-called intergenic region. According to a widely accepted model, the polymerase transcribes the template faithfully until it reaches the oligo-U signal. By a mechanism involving repeated cycles of backward slippage of the polymerase and the nascent strand in relation to the template, followed by elongation, the oligo-U stretch is thought to template the entire poly(A) tail of the mRNA, which finally comprises 100–300 Å residues. The polymerase then scans the intergenic region, probably without leaving the template, and resumes transcription at the downstream start consensus sequence. Capping of the downstream mRNA is intimately coupled with the transcription reinitiation process, since added preformed RNAs do not undergo this modification. Invariably, the junction between the 3' terminal leader template and the first gene (N, or NS1 in RSV) lacks an intergenic region and is defined merely by the transcription consensus start signal of the first gene.

Once recombinant systems allowing the experimental modification of *cis*-acting sequences became available, the function of the putative transcription signals was rapidly verified. Gene border sequences of rhabdoviruses and paramyxoviruses were shown to contain the signals directing the polymerase to accomplish the functions required for correct transcription of mRNAs, both in the virus context, and within bicistronic model genomes (see below), opening the way for expression of additional genes from recombinant RNP viruses (see design of vectors).

### *Means of Regulating Transcription*

The major element of transcriptional regulation is represented by the gene order and the polar transcription of genes. It is believed that the observed gradient of mRNAs is due to dissociation of polymerase complexes at each gene border, resulting in a progressive loss toward the 5' end. At each gene border of VSV, approximately one third of the polymerases that terminated an upstream mRNA fail to reinitiate transcription of the downstream gene (86). In contrast to VSV, whose intergenic regions consist of a conserved GA dinucleotide, rabies rhabdovirus exhibits intergenic regions of variable sequence and length (2, 5, 5, and 24 nts) (39, 176). Such a situation seems to provide a means for a more differentiated transcriptional attenuation of downstream genes. As revealed by Northern hybridization, a strikingly steep gradient in the abundance of mRNAs

was observed between the RV G and L genes, which are separated by the long 24-nucleotide intergenic region (40). In fact, a recombinant rabies virus in which the G/L gene border was replaced with the N/P border containing 2 nts transcribed significantly more L mRNA (64).

The length of individual intergenic regions varies considerably within the genomes of Rubulaviruses and pneumoviruses (1 to 56 nts). Interestingly, the analysis of the individual RSV pneumovirus gene junctions in bicistronic model genomes revealed no marked differences in initiation of the downstream reporter gene (98), suggesting that in contrast to rhabdoviruses, the length of the intergenic region is not a major means of determining reinitiation in this virus group.

The intergenic regions of paramyxoviruses and morbilliviruses comprise exactly the same length of 3 nts. Whereas a relatively smooth gradient of mRNA abundance is observed in measles virus, indicating equal probabilities of not reinitiating at each junction, in Sendai virus transcription larger disparities are found at the M-F and HN-L boundaries, showing that additional means exist to allow a virus to fine-tune the relative amounts of each gene product.

More drastic ways to down-regulate transcription of the 5' terminal L gene have been identified in rhabdoviruses and in RSV pneumovirus. The 3' non-coding region of the G gene of several cell culture-adapted rabies virus strains (ERA, HEP, and PV) contain a sequence mimicking a transcriptional stop signal recognized by approximately half of the polymerase molecules, resulting in the transcription of two differently sized G mRNAs (40, 121, 176). It is assumed that polymerase molecules terminating at the upstream signal are unable to reinitiate transcription at the far downstream (400 nts) L start signal, reducing L transcripts to approximately half of the standard amount. In the RSV pneumovirus, a unique situation for NNSV is observed. The M2 and L genes overlap such that the M2 stop/polyadenylation signal is situated downstream of the L start signal (37). This configuration may result in effective attenuation by two distinct mechanisms: First, initiation upstream of the termination signal should be considerably hampered. Second, most transcripts initiating at the L start signal terminate shortly thereafter at the M2 stop/polyadenylation signal (37). Transcriptional readthrough is thus obligatory for expression of the RSV L protein. Curiously, such an attenuation mechanism has not been described for the closely related pneumonia virus of mice (PVM), which otherwise exhibits the same gene pattern (143).

According to the mode of sequential synthesis, where transcription of downstream genes cannot be more abundant than that of the upstream gene, attenuation by enhancing the dissociation of polymerase affects transcription of all genes located downstream. Consistent with this view, this type of attenuation is found predominantly in the case of the L gene. A possibility to selectively

down-regulate expression of an internally located gene is transcriptional read-through, involving inefficient polyadenylation and termination. Specifically, some viruses read through some junctions at a higher frequency than usual and produce bicistronic mRNAs. Only the upstream ORF of such mRNAs is normally translated, whereas the downstream ORF is not expressed. Measles virus, for example, uses this mechanism to down-regulate M expression during persistent infections of the human brain, as found in patients suffering from SSPE (14). In addition, cellular factors might modulate the transcript gradients (151).

The majority of bicistronic mRNAs generated in natural rhabdoviruses result not from simply ignoring stop/polyadenylation signals but rather from a failure during polyadenylation and reinitiation. Both for VSV and rabies virus, bicistronic mRNAs were shown to contain internal poly(A) stretches (40, 84). An analysis of bicistronic M-G mRNAs of rabies virus revealed the presence of internal A-stretches of 40–100 residues, followed by the intergenic region complement (40). This indicates that polyadenylation by “stuttering” on the oligo U template is terminated before the complete poly(A) tail is synthesized. Without releasing the polyadenylated RNA, the polymerase appears to switch back to the colinear transcription mode.

#### *Directed Mutagenesis of Transcription Signals*

Analysis of mutant transcription signals within the context of a standard virus genome is hampered by the inherent feature of the stop-start transcription mechanism, where synthesis of a downstream mRNA depends on the polymerase having transcribed upstream genes. Therefore, reconstituted systems are preferred in which the sources for *cis*-acting RNP signals and the *trans*-acting functions required for transcription are separate. Model genomes are mostly used in combination with helper virus- or plasmid-expressed virus proteins to study the functions of modified signals. These model genomes contain two reporter genes separated by the gene border sequences to be analyzed.

In an RSV minigenome of this type, removal of the gene end signal of the upstream gene (the two genes were thus separated only by the original intergenic region nucleotides) resulted in readthrough, as expected. No initiation was observed at the internal gene start signal, confirming the importance of closely neighboring stop and start signals (99, 100).

Mutagenesis of the stop/polyadenylation signals of rhabdoviruses revealed that a U stretch of 7 residues, as is found in wt viruses, best supported polyadenylation and termination of the upstream transcript. In VSV, removal of a single U residue abolished the synthesis of the monocistronic upstream transcript completely (12, 85, 164). Interestingly, the resulting readthrough products contained exactly six templated A residues, demonstrating that the truncated U

stretch is not able to direct polymerase stuttering and suggesting that polyadenylation is required for termination and release of the RNA. The corresponding mutation in the RV stop signal did not abolish, but merely reduced expression of the downstream gene (S Finke, unpublished). A less dramatic effect showed the addition of U residues to the VSV signal; U stretches of up to 14 residues give rise predominantly to monocistronic mRNAs. These investigations demonstrated the important role of nucleotides upstream of the U tract and of the downstream intergenic region. Exchange of a C residue, which immediately precedes the U tract in many viruses, abolished termination in VSV completely (12), but again only reduced termination in RV (S Finke, unpublished). The downstream first residue of the intergenic region (G in all rhabdoviruses) is required for termination, while exchanges of the second intergenic region nucleotide were tolerated. The experiments also verified that polyadenylation and termination are independent of a downstream transcription restart signal and reinitiation, as could be assumed from the fact that the most 5' gene (L) of all NNSV is not followed by such a signal.

The requirements for successful reinitiation, however, are less well established. Transcription reinitiation seems to require upstream termination to occur within a short distance. Separation of stop and start signals of rabies virus by increasing numbers of residues (i.e. the enlargement of intergenic regions) results in decreasing transcription of the downstream gene. A distance of 24 nucleotides (as found in the RV G/L border) seems close to the allowed limit (S Finke, unpublished). Moreover, intergenic regions possessing the same length of 5 nucleotides, but different nucleotide composition of the three internal residues show marked differences in the readthrough rate (64). At least in rhabdoviruses, reinitiation requires a signal separate from the stop/polyadenylation signal: A VSV gene border mutated so that the start signal is directly adjacent to the U7 stretch of the stop signal (i.e. the stretch is A9) abrogated reinitiation of the downstream gene. A rabies virus start signal that overlaps the stop/polyadenylation signal (*italics*) but leaves both signals unchanged in sequence (..*CUUUUUUUUGURRNGA*..) is inactive in restart, but completely active in polyadenylation and termination (12, 65). This signal was used in a rabies virus ambisense gene expression vector to terminate transcription from the antigenome and to prevent the synthesis of antisense transcripts from virus genes (65).

The 3' terminal leader sequence is thought to play a crucial role in the control of viral gene expression. It is at least part of the polymerase entry site (see below) and separates the first gene (mostly N) from the 3' end of the genome. Synthesis of the leader RNA and initiation of the 3' proximal mRNA is different from transcription of the downstream mRNAs in various aspects. The leader RNA is not capped and does not possess a poly(A) tail. This is consistent with

the lack of the respective consensus signal sequences. The leader-N gene junction apparently does not contain nontranscribed intergenic nucleotides. Thus, termination of the leader RNA does not require the process of poly(A) stuttering, as does termination of mRNAs. In addition, the "restart" signal (which is identical to that of internal genes) need not be separated from the upstream termination site (the 3' terminal leader template base), as do internal rhabdovirus start signals.

The leader appears to carry an N-encapsidation signal, and the leader/N junction plays a crucial role in models toward elucidating the switch between transcription and replication (see below). Mechanisms should exist during transcription of NNSV that prevent encapsidation of virus mRNAs that are made for translation. Production of a leader RNA is perhaps necessary to get rid of terminal genome sequences carrying the encapsidation signal. For segmented viruses, which do not express a leader RNA, transcription of mRNAs may involve elongation of capped primers originating from cellular mRNAs ("cap-snatching") (94, 140), thereby differentiating their antigenomic full-length RNA from mRNAs. Both in segmented and nonsegmented viruses, transcription of the most downstream gene is always stopped preferentially at an internal transcription stop signal, thereby avoiding 3' ends identical to full-length antigenome RNA.

### *RNA Editing*

Members of the Paramyxovirinae subfamily are distinguished from other NNSV by a remarkable process of transcription known as RNA editing or pseudotemplated transcription (77, 95, 104). Interestingly, RNA editing is confined exclusively to the P gene. This mechanism, which allows access to overlapping reading frames, together with the use of alternative ribosome initiation, makes the "P" genes of Paramyxovirinae an extraordinary example of a virus gene compacting as much genetic information as possible into a single transcription unit.

These P genes contain a pyrimidine stretch, mostly U(5-6)C(3-7), at the start of the internal, overlapping V ORF. In addition to faithful copies of the template, mRNAs with extended G runs are transcribed from the genes, and the number of G insertions that occur for each virus group mirrors their requirement to switch into the out-of-frame ORF. In Morbilliviruses, a +1 frameshift is required to access the V ORF from the genome-encoded P ORF, and the insertion of a single additional G is the most prominent insertional event. In bPIV3, in which the V ORF and an additional third ORF overlap with the P ORF, one to six Gs are added in roughly equal amounts, so that mRNAs directing translation of all three ORFs are produced. For the rubulaviruses, interestingly, an insertional event is obligatory to produce the P protein while colinear transcripts of the P genes encode the V protein. Insertion of 2 G residues occurs at high frequency, gaining access to the remainder of the P ORF.

Since all NNSV that polyadenylate their mRNAs are thought to do so by reiteratively copying a short U stretch (followed by C, see above), it was suggested that the G insertions during P mRNA editing would similarly occur by pseudotemplated transcription. Indeed, introduction of the purine tract and sequences immediately upstream into the CAT gene of a synthetic Sendai virus minigenome allowed insertion of one or two G residues into the CAT gene-derived mRNAs during transcription by helper virus polymerase proteins (77, 130).

### *The Rule of Six in Transcription, RNA Editing, and Repair*

Evidence is now increasing that the rule of six plays an important role not only in replication, but may also have some impact on mRNA synthesis and P mRNA editing, and in diminishing the effects caused by "edited" genomes [for recent reviews see (77, 95)]. The role in mRNA synthesis is suggested by the observation that transcription signals of Paramyxovirinae appear to prefer certain hexamer positions, but the position varies among the genera. Most mRNA start positions of members from the paramyxovirus and rubula virus genera are in hexamer position 1, whereas morbilliviruses prefer position 2. Since the Paramyxo- and Morbillivirus intergenic regions separating the reinitiation signal from the stop/polyadenylation signal invariably contain three nts (mostly CTT), the conserved hexamer phase may be important for both termination and restart. The variability of rubulavirus intergenic region lengths suggests that in this genus the hexamer phase is conserved for mRNA initiation only rather than for polyadenylation/termination. Also, the P mRNA editing site (the first residue in the G run) is conserved with regard to a hexamer position, according to the virus group and the particular mode of editing, suggesting that the hexamer phase is important for the editing mechanism.

RNA editing was initially thought to occur exclusively during transcription of paramyxovirus mRNA, but recent experimental evidence suggests that under certain circumstances both insertion and deletion of nucleotides may also happen at the purine run of the P gene of Sendai virus during replication (77). Such a situation would imply that the integrity of genomes is affected. Intriguingly, all viruses that edit their P mRNA—and thereby possibly produce erroneous genomes—appear to adhere to the rule of six. Since efficient replication of such viruses requires that their genome lengths comprise exactly a multiple of six nucleotides, genomes that, by "RNA editing," have not introduced exactly six nucleotides would have a serious selective disadvantage, resulting in their rapid elimination (77, 95).

### *RNA Replication*

Replication of NSV genomes is a process quite different from transcription of mRNAs, although ostensibly performed by the same virus polymerase.

During replication, the polymerase acts in a processive mode in which the internal transcription signals are ignored. Moreover, the product of synthesis is not a free RNA, but an RNP with an encapsidated full-length RNA. In contrast to transcription, synthesis of RNPs (replication) requires an ongoing supply of N and P proteins. It is therefore assumed that polymerization and encapsidation are mechanistically linked (7, 67), and the polymerase involved could be regarded as an RNP-dependent RNP polymerase. Concurrent encapsidation of the growing chain involves the participation of preformed N-P and P-L complexes (82).

RNPs entering a cell are thus not able to replicate until transcription ("primary transcription"), and subsequent translation of mRNAs provides a critical amount of N and P protein. How the polymerase is then made to switch from primary transcription to replication is still a matter for speculation. The simplest model would be the existence of two functionally distinct forms of the polymerase, a nonprocessive transcriptase and a processive replicase, formed by alternative association with N and/or P proteins. There is so far no evidence that the RNP template itself could be modified by newly expressed proteins to determine the mode of RNA synthesis. The most widely accepted model to explain the switch states that newly synthesized N protein binds to nascent leader RNA and thereby prevents recognition of termination signals. This model is supported by the finding that newly synthesized N selectively encapsidates the leader RNA (15, 18, 29). A crucial site determining the further mode of synthesis appears to be the junction of the leader template and the first mRNA-directing gene. With enough soluble N-P complexes available, the leader junction signal is not recognized and replication proceeds. Successful reinitiation at this site, which should be possible in the absence of N protein, seems to definitely convert the polymerase to the nonprocessive mode in which elongation (transcription) is independent of assembly with N protein (177).

In principle, this represents an attractive self-regulatory mechanism; when N is limiting, the polymerase would be engaged predominantly in mRNA synthesis, raising the intracellular levels of virus proteins, including N. When N levels are sufficient, the polymerase would be switched to replication. Then, encapsidation, virus assembly, and budding would lower protein levels again. However, the reality might be more complex. With the availability of systems where replication of deficient genomes is supported by plasmid-expressed proteins, experiments have been performed to establish a correlation of N and P levels with a shift between transcription and replication. In several virus systems, this has not yet been substantiated, indicating that additional factors are involved (62, 180).

When the full-length antigenome RNP, which contains a faithful copy of the genome RNA, is completed, the antigenome RNP serves as a template for the

polymerase, although exclusively for replication. Synthesis starts at the 3' terminus of the antigenome, which is highly similar in nucleotide composition to the leader-specifying sequence of the genome RNA. A sequence corresponding to the leader stop/N-gene reinitiation signal is missing, however. The synthesis from the antigenome RNP of a leader-like RNA has been suggested. The function of such a (-) leader could possibly involve recycling of polymerase in the early stages of infection where insufficient soluble N protein is available, and reduce unsuccessful replication attempts (104). Synthesis of discrete free RNAs from antigenome RNP has not been demonstrated in any natural NNSV. The newly synthesized genome RNPs may then serve again as templates for both transcription ("secondary transcription") and replication. In all stages of the virus' life cycle, transcription remains by far the dominant mode of RNA synthesis. Replication gives rise to huge amounts of genome over antigenome RNPs. The ratio of genome and antigenome RNPs appears to be directed solely by the different replication activities of the genome and antigenome promoters (see below).

#### *Promoters for Replication and Transcription*

Early studies of small RNAs of defective interfering (DI) particles, which can be replicated in the presence of nondefective helper virus, indicated that the *cis*-acting signals required to direct encapsidation, replication, and transcription of NNSV are contained in the 3' terminal sequences of the genome and the antigenome (17, 106, 108, 137). More recently, this finding was verified for many viruses by taking advantage of the systems that allow recovery of replicatable model genomes from cDNA (26, 36, 42, 55, 129, 131, 160, 163, 181). The identity of the primary sequences engaged in common functions during replication, such as polymerase binding, replication initiation, encapsidation, and elongation, is still poorly defined; so these RNA ends are presently regarded as the "genomic promoter" and the "antigenomic promoter," respectively (28).

A canonical feature of both defective and nondefective NSV genomes is the complementarity of 3' and 5' terminal nucleotides. Exactly 12 nts in paramyxoviruses and 11 nts in rhabdoviruses are identical in the genome and antigenome RNAs. For the segmented influenza A virus, base-pairing of the complementary ends has been demonstrated physically, and formation of defined panhandle structures was shown to be required for promoter function. However, such an interaction is not observed in NNSV (111), nor is it likely, since bases in the RNP structure are not exposed to the surface, as described for influenza virus (13). Rather, the identical ends of the genome and antigenome RNA represent important sequences involved in encapsidating the newly synthesized leader RNAs by N protein and initiating RNA synthesis.

For both VSV and Sendai virus, binding of N protein to the terminal nucleotides of the leader RNA occurs *in vitro* (15, 123), whereas a more internal encapsidation site was suggested for rabies virus (184). Such experiments may lead to the delineation of N-binding RNA sequences as well as of RNA-binding N domains of different viruses. They may also prove useful in understanding the mechanisms involved in "physiological" encapsidation of NNSV, which involves polymerization and requires the help of the polymerase. "Illegitimate" encapsidation of naked T7 RNA transcripts by N protein in the cytoplasm of transfected cells is extremely inefficient, but just sufficient to transform some transcripts into an RNP-like structure that is suitable as a template for the polymerase. This has been the clue to recovering NNSV genome analogs from cDNA. *Cis*-acting sequences specifying the physiological "encapsidation site" might also include the sequences acting as polymerase binding/entry site, and signals for replication initiation, and elongation. As the rule of six implies, these signals may not only consist of *cis*-acting RNA sequences but may also require specific N domains associated appropriately (i.e. in the correct hexamer phase) with defined RNA bases. Thus, only sophisticated experimental strategies are suitable to dissect particular *cis*-functions of the NNSV "promoters."

The approximately 50 nt-long noncoding sequences following the 3' terminal identical nt-stretches of the genome and antigenome RNA do not exhibit well-conserved sequence motifs, but are similar in possessing a high content of A and U residues. These short terminal stretches seem to be sufficient to confer a high replicative activity to minigenomes of all NNSV. In the rhabdovirus VSV, DIs have been identified that possess the terminal 45 nts of the antigenome (119), and artificial genomes with the 51 3'- and 46 5'-terminal nucleotides replicated efficiently (131). In Sendai paramyxovirus, as few as the terminal 31 nts from the antigenome were initially thought to be sufficient for promoting replication, independently of the adjacent sequences (173). However, repetitive sequences located 80 nucleotides from the ends (i.e. within the N or L gene) have now been identified in the paramyxoviruses Sendai virus and SV5, whose presence enhances replication of defective interfering RNAs manifold (125, 172). Thus the paramyxovirus promoters may not be confined to the leader template regions, as seems to be the case for rhabdoviruses.

The signals that render the genomic promoter transcriptionally active, and which in this respect distinguish it from the antigenome promoter, have not yet been delineated. However, one particular region of the genomic promoter clearly is critical for initiating the transcription mode. At least part of the putative *cis*-acting signal enabling the synthesis of discrete downstream mRNAs is the junction of the leader template sequence and the first protein-encoding cistron, containing the consensus transcription start signal (177). Indeed, the 3' terminal part of the Sendai virus leader-coding region can be replaced by

the corresponding sequence of the antigenomic promoter without significantly affecting transcription of mRNAs (28, 71).

The replication activities of the genome and antigenome promoters are different, but the sequences responsible have not yet been identified. In all NNSV, the antigenome promoter (the 3' end of the antigenome) is considered to be the stronger replication promoter. This has been made responsible for the observed preponderance of genome strand-containing RNPs over antigenome-containing RNPs in infected cells. The bias is moderate in paramyxoviruses like Sendai virus, where 10–40% of RNPs may contain antigenome RNA (93). An extreme bias is reported for rhabdoviruses. As determined in rabies virus-infected cells, only 2% of RNPs contain antigenome RNA (65).

The high replication activity of antigenome promoters is, in most cases, the major factor for the observed interference of defective RNAs with replication of nondefective viruses. The overwhelming majority of natural defective interfering (DI) RNAs of VSV and paramyxoviruses lack the transcriptionally active genome promoter (17, 106, 108, 137). These so-called "copy-back" DI RNAs have replaced the genomic promoter with sequences complementary to the parental 5' ends, i.e. the antigenome promoter. They are incapable of transcription but interfere significantly with replication of nondefective helper virus and are efficiently assembled into virions (137). Copy-back DIs are thought to be generated by a process in which the polymerase leaves the template RNP while carrying the nascent chain. Resuming polymerization "backwards" on the newly synthesized strand would then lead to an RNA possessing complementary ends (60). The resulting presence of two identical strong replication promoters should enable such DI RNAs to efficiently outcompete replication of the helper virus, which has one strong promoter and one weak promoter. In contrast to 5' copy-back DIs, which originate from genome synthesis, natural defective RNAs with identical ends derived from the weak genomic promoter (3' copy-back RNAs) have not been identified.

The above observations have been used in support of a sequence-dependent, intrinsic replicative advantage of the antigenome promoter over the genome promoter. However, the same effect could be expected in competition between transcription and replication at the genome promoter. In all stages of the virus' life cycle, transcription is the predominant mode of RNA synthesis. If nonconditioned polymerase molecules entering at the genomic promoter are recruited for either replication or transcription, it would be at the expense of replication. There is one report so far describing how the balance between replication and transcription can be altered by modifying the terminal *cis*-acting sequences, namely, by extending the complementarity of terminal stretches in artificial VSV minigenomes (181). The assumption that particular antigenome promoter sequences confer an intrinsic advantage in replication has only recently

been given more substance. Calain & Roux (28) identified a transcriptionally active Sendai virus DI RNA possessing a chimeric genomic promoter, in which the terminal 42 nts had the sequence of the antigenomic promoter. Although transcriptionally active, the DI interfered with helper virus as do nontranscribing copy-back DIs, indicating that transcription does not necessarily interfere with replication. Upon introduction into a full-length Sendai virus cDNA, the resulting nondeficient virus was also found to interfere with replication of wt Sendai virus (71). Findings obtained with a bicistronic model genome of human respiratory syncytial virus (RSV) also support a lack of competition of transcription with replication. Upon deletion of the transcriptional start signal of the 3' proximal gene in a model genome, transcription of a reporter gene was abolished. However, augmentation of replicative antigenome synthesis could not be demonstrated (100).

A different approach toward establishing functions and activity rates of rhabdovirus promoters was applied in our laboratory for rabies virus. The lack of natural 3' copy-back DIs prompted us to investigate whether it is possible to generate a RV whose replication and transcription is directed exclusively by transcriptionally active "genomic" promoters. In a full-length RV cDNA, the virus 5' terminal sequences were replaced with sequences specifying the genomic promoter, including the leader/N junction, and an adjacent CAT reporter gene (65). The recovered virus propagated autonomously, showing that the antigenomic promoter of rabies virus does not contain any function that would not be performed by the genomic promoter sequences as well. Indeed, a CAT mRNA was transcribed from the antigenome RNP by sequences corresponding to those directing N mRNA transcription from the genome RNP. The replacement of the antigenomic promoter also equalized the relative levels of genomic and antigenomic RNA in infected cells, confirming the assumption that the terminal promoters of the viral RNAs alone are responsible for adjusting the appropriate balance of genome and replicative intermediate. Interestingly, the equal amounts of RNAs resulted in the production of equal amounts of virions containing genome or antigenome RNPs, showing that there is no mechanism for selective incorporation of negative-strand RNPs into virions.

In contrast to the rhabdovirus VSV, where no antigenomic RNPs could be found in virions (9), the assembly of antigenome RNPs into virions has been reported for Newcastle disease virus (147) and Sendai virus (92). The ratios of Sendai virus genome and antigenome were nearly identical in virus-infected cells and in virions (93, 122). In contrast to rhabdoviruses, however, polyploidy is common in the pleiomorphic Sendai virus, and a less stringent incorporation of positive RNPs might not have deleterious effects on the formation of infectious virions.

The most unexpected finding with the 3' copy-back rabies virus, whose replication is directed by two "weak" promoters, was a very high replicative activity.

Total RNP synthesis in infected cells was only reduced to 60% as compared to standard virus. When the ratios of template and replicative products are also taken into account, the replication activity of the genomic RNA promoter had been raised by a factor of approximately 30, whereas the transcription rate was apparently unaltered. Obviously, the genomic promoter is only weak in competing with the antigenome promoter sequence (65). Subsequent experiments, in which the levels of available polymerase were augmented, indicated that the limiting factor for replication is the polymerase itself, rather than the potency of the promoters (64).

## GENETIC ENGINEERING OF NNSV

### *Technical Aspects: Reconstitution of RNPs*

Genetic manipulation of RNA viruses requires the ability to recover the virus from a DNA copy of the RNA genome. This possibility came early with positive-strand viruses (144, 169), where the genomic RNA itself, or a corresponding DNA-derived transcript, acts as mRNA; and is infectious after introduction into a suitable host cell [for review see (19, 20, 41)]. The minimum infectious unit of NSVs is the RNP, whose assembly in nature is RNP dependent. All efforts toward manipulation of NSV, therefore, were directed toward "illegitimate" assembly of RNA into RNP-like structures.

The initial breakthrough came with the description by Palese and colleagues of a system that allowed generation of biologically active Influenza A virus RNPs containing artificial RNA (110). Transcripts generated *in vitro* possessing authentic terminal sequences from an influenza genome segment and containing an internal CAT reporter gene were encapsidated *in vitro* by purified influenza virus nucleoprotein (NP), and the viral polymerase proteins (PA, PB1, and PB2). After transfection of the reconstituted RNP into virus-infected cells, the construct was replicated and transcribed. Reassortant virus containing a recombinant RNP as one of the 8 genome segments can be obtained after selection (61). This system has since been extremely useful for the analysis of influenza virus (70, 128).

However, all attempts to produce active RNPs of nonsegmented virus systems *in vitro* failed, most likely owing to the tighter RNP structure (13). Krystal and colleagues first demonstrated that a short artificial RNA construct inside a cell could be rescued into a form that allows recognition and amplification by the polymerase of Sendai virus (129). The synthetic RNA was generated *in vitro* by T7 RNA polymerase transcription from a linearized plasmid to create precise ends. It corresponded to a Sendai virus minigenome in which the entire coding region was replaced with the coding region of the CAT reporter gene. After transfection of the *in vitro* transcribed RNA into cells, CAT activity was observed after subsequent infection of the cells with Sendai virus. The artificial

RNPs were also packaged into virus particles that could be passaged in cell culture in the presence of helper virus. Helper virus-driven rescue of transfected RNAs was also successful in other paramyxovirus systems such as RSV (36), PIV-3 (51, 55), and measles virus (160), but, interestingly, not yet for any standard rhabdovirus.

The replacement of helper virus by proteins expressed from transfected plasmids with T7 promoters in cells infected with a recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3) (66) markedly enhanced recovery rates and allowed for investigation of viral *trans-acting* factors required for replication, assembly, and budding of virus-like particles (49, 133).

Since the sequence corresponding to the viral 3' terminus has to represent the utmost end of the model genome transcripts to be functional (36, 51, 132), the development of plasmid vectors designed to yield RNAs with discrete 3' termini inside a cell represented a major step forward technically. This was achieved by exploiting the autolytic activity of ribozyme sequences, as first successfully used for intracellular generation of functional nodavirus RNA (4) and VSV RNAs (132). This system allowed much more efficient production of appropriate RNA inside a cell, as compared to transfection by RNA transcribed *in vitro* or transfection by linearized DNA constructs to obtain intracellular runoff transcripts. The latter especially is not very effective in the presence of vaccinia virus, most likely owing to ligation and modification of DNA by vaccinia virus enzymes.

The antigenome ribozyme sequence of hepatitis delta virus (HDV) has generally been used; this has the advantage of requiring only sequences downstream of the cleavage site for autocatalytic activity and apparently being indiscriminate with regard to upstream sequences (138). RNAs ending with the correct 3' nucleotide can be generated by autolytic cleavage from primary transcripts containing the HDV ribozyme sequence immediately downstream of the viral sequences. According to the ribozyme cleavage mechanism, the 3' terminal ribose of the (upstream) genome analog should possess a cyclic 2'-3' phosphate instead of a hydroxyl group (151). This modification might contribute to the success of the approach, in preventing polyadenylation of the RNA by vaccinia virus enzymes (72), for example, or in delaying degradation of the RNA 3' terminus.

The genome analogs widely used in the different virus systems mimicked natural short 5' copy-back DI RNAs, or corresponded to transcriptionally active internal deletion type minigenomes, possessing the virus genome- and antigenome-promoter sequences, and encoding one or more reporter genes [reviewed in (38)]. While these systems work in principle for all NSV, including segmented bunyaviruses (56, 109), species-specific modifications are required in some cases. For instance, while the entire rhabdovirus life cycle, including assembly and budding of virions, is correctly performed in the presence of

vaccinia virus, Sendai paramyxovirion assembly is killed by vaccinia (27). With "editing" paramyxoviruses obeying the rule of six, the transcripts must comprise an appropriate number of nucleotides. Although the initial step of illegitimate encapsidation appears to be equally efficient with nonhexamer RNAs, the subsequent amplification by the virus polymerase is much more efficient for an RNA with the appropriate length. Also, inactivation of V- and C-protein frames of support plasmids providing P may increase amplification of rescued RNAs (25, 185).

In addition to the set of N, P, and L proteins, efficient transcription from pneumovirus genome-analogs requires the presence of the M2-1 transcription elongation factor (35, 73). This protein must therefore be included in critical experiments such as recovery of full-length infectious virus and analysis of transcription signals.

#### *Think Positive: Recovery of Infectious Viruses from cDNA*

Although the requirements for rescuing cDNA-derived RNAs of NNSV into RNP s have been determined in the minigenome systems, it took some time before the first complete infectious NNSV, the rabies rhabdovirus, was recovered from cDNA (157). The key to reproducibly recovering recombinant virus was the use of a plasmid directing transcription of antigenome (positive-strand) RNA, rather than genome RNA. The use of positive-sense RNA turned out to be successful for other NNSV as well, and established systems for recovering members from all rhabdovirus and paramyxovirus genera are now available (Table 1).

The advantage of using positive-strands to encapsidate full-length virus RNA appears obvious. If naked negative-strand RNA genomes are produced in the cytoplasm of cells that are also producing complementary mRNAs encoding viral proteins, the two can hybridize and prevent the critical assembly of the genome into the RNP. In starting with an antigenome RNP, only one successful round of replication driven by the plasmid-encoded support proteins is required to yield an infectious genome RNP.

The magnitude of the antisense problem is illustrated in work from a group in Japan. Nagai et al reported recovery of Sendai virus even when starting with the negative-strand, albeit at very low efficiency (91). Efficiency with the positive-strand was in the range of one recovery per  $10^4$ - $10^5$  transfected cells, the highest efficiency yet reported for any NNSV, but recovery with the negative-strand construct was 100-fold lower. Since negative-strand RNA was synthesized much better in this system than the positive-strand, the difference is probably even greater than 100-fold.

Intracellular assembly of positive-strand RNAs with plasmid-expressed proteins is also applicable to segmented NSV. Recently, Elliott's group (21)

reported the recovery from cDNA of Bunyamwera virus. In this case, six different plasmids were used to produce RNA transcripts and support proteins. Three plasmids yield the three Bunyamwera antigenome RNAs (L, M, and S segments). As in NNSV, these antigenomic RNAs are unable to express virus proteins. To supply all the proteins necessary to induce an infectious cycle, three other plasmids encoding Bunyamwera polymerase, nucleocapsid proteins, membrane glycoproteins, as well as two nonstructural proteins of unknown function were transfected, resulting in the recovery of virus.

The authenticity of recovery systems is thus now well established. The protocols used differ mainly in how T7 RNA polymerase or support proteins are produced. Vaccinia virus vTF7-3 is generally used; which requires that the rescued virus be separated from vaccinia virus. This separation is easily accomplished by physical (105, 157) or biochemical means (78, 81, 182), or by passage in cells that are not permissive for vaccinia virus (21, 71). Alternatively, a host range-restricted vaccinia recombinant (MVA-T7) is used that expresses T7 polymerase (166), but does not replicate in many mammalian cells (11, 34, 150). Finally, vaccinia virus-independent systems have been developed by generating appropriate cell lines constitutively expressing T7 RNA polymerase alone (64) or together with helper proteins (145, 183). Recovery rates can be augmented to a certain extent by such refined protocols, but not by orders of magnitudes. In optimized protocols, rates of about 1 recovery in  $1 \times 10^6$  cells for rhabdoviruses and  $10^5$  for paramyxoviruses can be expected, making engineering of NNSV laborious. The intrinsic bottleneck is caused by the inefficiency of "illegitimate" encapsidation and is experienced in all NNSV systems. However, since the systems are independent from homologous helper viruses, there is no need for selection procedures. Thus, even the isolation of weakly growing virus mutants is feasible.

### *NNSV as Expression Vectors*

The possibility of genetically manipulating NSV opens many aspects of virus biology and virus-host interaction for study. Any mutation, including those studied previously in other contexts, can now be examined in the most relevant and important context, the virus' life cycle. Analysis of numerous mutations with the infectious clones will probably also identify mutations that attenuate the viruses and that could be used to develop new vaccine strains. Apart from the impact for study of the particular virus system, the possibility of generating recombinant NNSV may find valuable application in basic and applied fields of biology and medicine.

NNSV have an exciting potential as vectors to express foreign genes. Their capacity to express additional protein sequences or transcription units has been amply demonstrated. A major problem experienced with other RNA virus

vectors has been the low integrity of foreign or nonessential sequences. This is due both to error-prone RNA synthesis by RNA-dependent RNA polymerases, which lack proof-reading activity; and to recombination, by which sequences not strictly required for efficient virus propagation are rapidly eliminated [for a recent discussion see (120)].

An early intimation that NNSV are quite different in this aspect was the identification of inessential genetic material in rhabdo- and paramyxoviruses. Large noncoding regions present in the G gene of RV (3' terminal) or in the F gene of measles virus (5' terminal) could be deleted with no obvious effect on virus replication (146, 157). The following experiments on expressing foreign genes and altering the genome structure of NNSV confirmed that the sequences of cDNA rescued into a recombinant virus are highly stable in the absence of selection both in cell culture and in infected animals (23, 118, 153, 154).

As evident from the formation of defective interfering RNAs, recombination also happens in nonsegmented negative-strand RNA viruses; however, this event is by several orders of magnitude less frequent than in most positive-strand RNA viruses. Moreover, the events leading to DI RNAs could exclusively represent intramolecular recombination (106, 119). There is no experimental evidence for recombination between two NNSV RNPs, although the differences in gene order of natural NNSV or the appearance of novel genes must have resulted from recombination (143, 175). Most likely, the tight RNP structures of NNSV and the structure of the active polymerase complex reduce the possibility of recombination. From this point of view, NNSV appear well suited compared to vectors based on various positive-strand RNA viruses. Moreover, the integrity of their RNPs, and the cytoplasmic replication of NNSV should prevent reverse transcription and unintended integration of viral cDNA into the cell DNA when applied in approaches toward transient gene therapy.

### *Design of Viral Vectors*

The modular nature of their genomes makes it easy to engineer additional genes, in the form of an extra cistron, into an NNSV. The introduction of a construct containing the desired open reading frame and an upstream consensus gene border, including the polyadenylation/stop signal, intergenic region, and transcription restart signal, results in faithful transcription of an additional, polyadenylated mRNA (23, 78, 96, 118, 157). The downstream noncoding region of the G gene has generally been used to insert additional cistrons into rhabdoviruses (96, 118, 157). Bukreyev et al introduced a CAT gene between the RSV G and F genes (23). It seems to verify that foreign genes can occupy positions not only between the virus genes, but also on 3' and 5' proximal positions. In a recombinant Sendai virus, the firefly luciferase gene was introduced between the leader RNA and the N gene. This resulted in high expression of luciferase.

Adjusting the rate of foreign gene expression by selecting a more up- or downstream location is an attractive tool. However, each extra gene border attenuates the transcription of all downstream virus genes by partial dissociation of the polymerase (see above). Expression of the 3' distal L polymerase will therefore probably become a limiting factor after genes are integrated in upstream positions, and especially when multiple extra cistrons are to be introduced. Although a single CAT gene upstream of L had no obvious effect on RV and VSV propagation (118, 154), a 20-fold decrease of virus titers was observed after a CAT cistron was introduced between the G and F genes of RSV (23). Expression of luciferase as the 3' proximal gene of Sendai virus was accompanied by reduced plaque sizes, slower replication kinetics, and several-fold decreased yield of the virus (76). The insertion site for a gene to retain reasonable virus amplification combined with most proficient gene expression has to be determined empirically. In addition to selecting the position of foreign genes, expression levels are being further fine-tuned by using modified transcription signals that differ in their ability to direct restart or readthrough, as described above.

Although the gene order of natural NNSV is highly conserved, the gene order of rhabdovirus vectors can be altered extensively, yielding artificial viruses. This has been shown for VSV in which the location of the internal three genes P, M, and G was changed to all possible combinations. In addition, VSV recombinants have been recovered in which the N gene was moved successively downstream from the parental 3' proximal position toward a position between G and L (180). Although the natural gradient of mRNA and protein levels was lost, to varying degrees, most of these VSV recombinants grow remarkably well in cell culture. In mice they show an attenuated phenotype, but replication is high enough to induce protection against wt VSV challenge (180). In our lab, two deficient rabies viruses, one encompassing the N, P, and L genes, and the other the envelope proteins M and G, complemented each other to yield a bipartite virus vector, which is not able to kill mice, but induces protection (T. Mebatson, in preparation).

The linear relationship between the length of the helical RNPs and the particle size (at least in rhabdoviruses) suggests that there might be only minimal constraints on the amount of additional RNA in this virus group. This makes recombinant NNSV promising candidates for the expression of large genes, and of multiple genes. With regard to the latter, limitations are expected due to transcriptional attenuation of virus genes by polymerase fall-off at gene borders. To surmount this problem, the foreign genes may be expressed from a 5' proximal location, downstream of L, but at the expense of expression level.

A promising way to circumvent transcriptional attenuation of virus "house-keeping" genes while allowing high-level expression of multiple foreign genes

is provided by artificial viruses similar to the ambisense rabies virus described above, which contains a transcriptionally active promoter in both the genome and antigenome RNA (65). It seems possible to recruit the antigenomic RNP as a template for typical stop/start transcription of several additional genes with the same efficiency as virus genes are expressed from the genome RNP. Moreover, further manipulation of the viral promoters in a way that the ratio of transcription and replication can be modified or the use of chimeric promoters that combine transcription activity of the genome promoter with the high replicative activity of the antigenomic promoter (28, 71, 181) should yield ambisense viruses with the desired characteristics.

Finally, NNSV carrying novel proteins in their envelopes may have a role to play as particulate vaccines, and as targetable gene delivery vectors. Many paramyxoviruses and VSV may accommodate glycoproteins from other viruses, after mixed infections of cells, a process called pseudotyping (186). The expression from recombinant VSV of a variety of foreign virus surface proteins such as measles virus F and H or influenza A HA confirmed that they are incorporated efficiently into virus particles in addition to the VSV G (96, 153). Such viruses with a "mosaic" surface may be valuable for inducing immune responses to both parental viruses.

Analysis of rabies virus deletion mutants lacking the M or the G genes revealed that virus budding from the cell surface membrane is driven by the M protein layer surrounding condensed RNPs (T Mebatsion, submitted; 116). This is similar to the situation whereby retrovirus particles are formed by the MA protein [for review see (107)]. In the absence of G, enveloped rhabdovirus particles are released that do not possess a virus surface protein and therefore are unable to enter a cell (116). This situation was predicted to allow for generation of rhabdoviruses in which their own spike protein is replaced with a foreign glycoprotein. Consequently, the cell tropism of the novel virus would be determined exclusively by the receptor specificity of the introduced glycoprotein.

The feasibility of this approach has been demonstrated recently. Rhabdovirus particles carrying the Env-protein of HIV selectively infect cells displaying the appropriate HIV-receptors (87, 114). Surprisingly, the requirements for successful incorporation of envelope proteins have turned out to be different for rabies- and VSV-derived particles. While proteins require a specific cytoplasmic tail for selective incorporation into rabies virus-derived envelopes (114, 115), VSV particles incorporate proteins indiscriminately (96, 153, 156) provided that a short nonspecific cytoplasmic tail is present (152).

In another set of experiments exploiting the propensity of rhabdoviruses to incorporate membrane proteins, the specificity of virus spike/receptor interactions was first used in reverse to generate virus vectors that selectively target

cells infected with another virus. Rabies virus- and VSV-derived particles with incorporated HIV receptor complexes such as CXCR4/CD4 (115, 156) or CCR5/CD4 (KK Conzelmann, unpublished data) selectively entered cells displaying T cell-line tropic and macrophage-tropic HIV Env spike protein, respectively, on their surfaces. In this novel situation, it is not a virus-protein that mediates the fusion of virus- and cell membranes, but rather the Env-protein on the surface of HIV-infected cells. Actually, the "anti-viruses" represent deficient viruses. They possess cell membrane proteins (CXCR4, CCR5, and CD4), taking over the function of a virus attachment protein, but they lack their own fusion protein. This defect is "complemented" only by HIV-infected cells, which provide the necessary fusogenic protein. It is possible to generate HIV-infected cell-dependent rhabdovirus-like agents that carry the relevant HIV-receptor/coreceptor genes in their genome (156; KK Conzelmann, unpublished data). Moreover, with such agents there is a possibility of expressing cytopathic genes or anti-HIV reagents. Whether these agents help to destroy HIV-infected cells and to reduce HIV-load *in vivo* awaits confirmation.

Exchange of surface proteins is also possible with paramyxoviruses. As previously shown for VSV and rabies virus, where glycoproteins from different serotypes are exchangeable without affecting virus growth to any extent (105, 117, 182), this is also possible with Parainfluenza virus types 1 and 3 (170). Recombinant measles-like virus in which the F and H genes were replaced with the G gene of VSV rhabdovirus, or a gene encoding a chimeric G protein possessing the cytoplasmic tail of F, was genetically stable (161). The formation of chimeric viruses, however, was substantially delayed and the titers obtained were reduced about 50-fold as compared to standard measles virus. These experiments suggested a less crucial role for measles M in virus formation and budding as compared to rhabdovirus M proteins, since the VSV G-containing measles-derived virus seemed to lack M protein, whereas the virus containing the chimeric G/F protein incorporated M protein into particles. Mice immunized with the chimeric viruses were protected against lethal doses of wt VSV.

## PERSPECTIVES

The manifold possibilities of manipulating gene expression and structure of NNSV predict the future design and recovery from cDNA of many novel composite NNSV viruses, combining the desired envelope features of one virus with the desired gene expression machinery of another. The relative ease and the degree by which NNSV allow their cell tropism to be manipulated to deliver genes only to the target cells of interest is unprecedented by other virus vectors. There is thus great potential for developing improved attenuated vaccines

and targetable gene delivery vectors for purposes such as transient gene therapy. Transient expression of genes may well be helpful for conditions such as cystic fibrosis or cancer therapy and the use of RNA viruses may have advantages over using viruses with a DNA phase. There is the option of simply exchanging immunogenic surface proteins, which is valuable where repeated administration of vectors is required. NNSV are available with a wide spectrum of phenotypes, ranging from highly cytopathic agents to those that induce persistent infection. This virus group therefore constitutes an exceptional pool from which to generate biomedical tools, including antiviral agents.

In view of the apparently low rate of genetic exchange between natural RNP viruses, the tools now available will bring about virus types that never existed before in nature. Although it is predicted that the overwhelming majority of such artificial viruses will be unable to compete with naturally selected viruses in any respect, nevertheless, it is imperative that appropriate care be taken, as with chimeric DNA and positive-strand RNA viruses.

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